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Naf1p is a box H/ACA snoRNP assembly factor

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ABSTRACT

Box H/ACA small nucleolar ribonucleoprotein particles (snoRNPs) contain four essential proteins, Cbf5p, Gar1p, Nhp2p, and Nop10p, each of which, with the exception of Gar1p, is required for box H/ACA snoRNA accumulation. Database searches identified a novel essential protein, which we termed Naf1p, with a region of homology to the RNA-binding domain of Gar1p and other features in common with hnRNP-like proteins. Naf1p is localized to the nucleus and is not a stable component of the H/ACA snoRNPs, but it is required for the accumulation of all box H/ACA snoRNAs. This requirement is not at the level of snoRNA transcription initiation or termination. Naf1p shows in vitro RNA-binding activity and also binds directly to Cbf5p and Nhp2p. Naf1p was shown to bind to the CTD in vivo in a two-hybrid assay, and the phosphorylated CTD, but not the nonphosphorylated CTD, was shown to precipitate tagged Naf1p from a cell lysate. We propose that Naf1p is recruited to the CTD of RNA polymerase II and binds to nascent box H/ACA snoRNAs promoting snoRNP assembly.

Keywords: ACA snoRNPs; box H; snoRNP assembly

INTRODUCTION

Ribosomal RNA modification and processing requires two large families of small nucleolar RNAs (snoRNAs), which are associated with protein components in small nucleolar ribonucleoproteins (snoRNPs). The box C/D snoRNPs select sites of rRNA 2′-*O*-methylation, whereas the box H/ACA snoRNPs select sites of pseudouridine formation (reviewed in Tollervey & Kiss, 1997; Weinstein & Steitz, 1999; Kiss, 2002). In addition, a few members of each class are required for correct processing of the pre-rRNA to the mature rRNA. In yeast, these include the box H/ACA snoRNAs snR10 and snR30, both of which are required for normal processing on the pathway of 18S rRNA synthesis (Tollervey, 1987; Morrissey & Tollervey, 1993). The box H/ACA snoRNPs contain four common proteins, Nhp2p, Nop10p, Gar1p, and the pseudouridine synthase Cbf5p. Each of these proteins is required for H/ACA snoRNA accumulation, with the notable exception of Gar1p (Girard et al., 1992; Henras et al., 1998; Lafontaine et al., 1998; Watkins et al., 1998).

Many snoRNAs have an unusual biosynthetic pathway, being excised from the introns of pre-mRNAs. In most cases, the exons of these pre-mRNAs encode proteins, which generally have some role in ribosome synthesis or function (Kiss, 2002). The yeast H/ACA snoRNAs are rather unusual in being predominately synthesized from independent transcription units, and only snR44 is known to be intron encoded. Despite the diversity of genomic organization, in all cases, snoRNA synthesis relies on the generation of entry sites for exonucleases in the precursor molecule followed by exonuclease trimming to produce a mature snoRNA. Concomitant with the processing of precursor molecule, snoRNP-specific complexes assemble on snoRNA sequence and protect it from further exonucleolytic digestion (Filipowicz & Pogacic, 2002).

During the synthesis of yeast snRNAs and snoRNAs, a hnRNP-like protein, Nrd1p, is proposed to bind to nascent transcripts, recruiting another RNA-binding protein, Nab3p, and the putative RNA helicase Sen1p, and promoting transcription termination (Steinmetz et al., 2001), probably via interactions with the C-terminal domain of the largest subunit of RNA polymerase II (RNA pol II; Yuryev et al., 1996; Conrad et al., 2000).

In vertebrate cells, the assembly of snRNPs in the cytoplasm and snoRNPs in the nucleus are likely to be mediated by a large oligomeric complex, termed the SMN complex, consisting of the SMN (survival of motor neurons) protein together with the Gemin2 to 6 proteins, and probably other factors (Fischer et al., 1997; Charroux et al., 1999, 2000; Pellizzoni et al., 2001a, 2002; Gubitz et al., 2002). In the nucleus, the SMN

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complex also associates with RNA pol II (Pellizzoni et al., 2001b). SMN binds directly to the glycine and arginine-rich repeat domains (RGG or GAR domains), which are present in the Sm protein component of the snRNPs, in fibrillarin, the box C/D snoRNA-associated 2′-*O*-methylase, and in hGar1p (Brahms et al., 2001; Jones et al., 2001; Meister et al., 2001; Pellizzoni et al., 2001a). However, although the snoRNP and Sm proteins are highly conserved in the evolution, no clear homologs of the SMN complex have been identified in *Saccharomyces cerevisiae* or in plants.

Database searches identified Naf1p, a novel protein with homology to both Gar1p and hnRNP-like proteins. Naf1p is required for accumulation of H/ACA snoRNAs and may bind to the same site as Gar1p in the snoRNA and participates in snoRNP assembly.

RESULTS

Naf1p is a novel yeast hnRNP-like protein

We reasoned that novel proteins involved in yeast snoRNP assembly could be detected by their relationships to known snoRNP proteins and/or protein involved in snoRNP synthesis. FASTA searches (Pearson & Lipman, 1988) of the yeast proteome identified a largely uncharacterized, essential ORF (YNL124w) encoding a protein with a region of significant homology (32% identity, 49% similarity) to the RNA-binding domain of the box H/ACA snoRNP protein Gar1p1p (Bagni & Lapeyre, 1998) (Fig. 1A,B). We termed this protein nuclear assembly factor 1 (Naf1p). Sequence comparisons against the yeast proteome often produce inflated and unrealistic estimates of statistical significance due to the small database size, and we sought confirmation of the Naf1p–Gar1p relationship by iteratively searching the nonredundant protein database. Because of its high compositional bias, Naf1p sequence was processed with SEG (Wootton & Federhen, 1996) to remove low-complexity regions and to delineate three globular regions that were used individually for further sequence analysis. PSI-BLAST searches (Altschul et al., 1997) with the Naf1p domain encompassing residues 110–220 readily identified orthologs in *Schizosaccharomyces pombe* and several metazoans (Fig. 1C). Reciprocal searches with Naf1p orthologs confirmed the

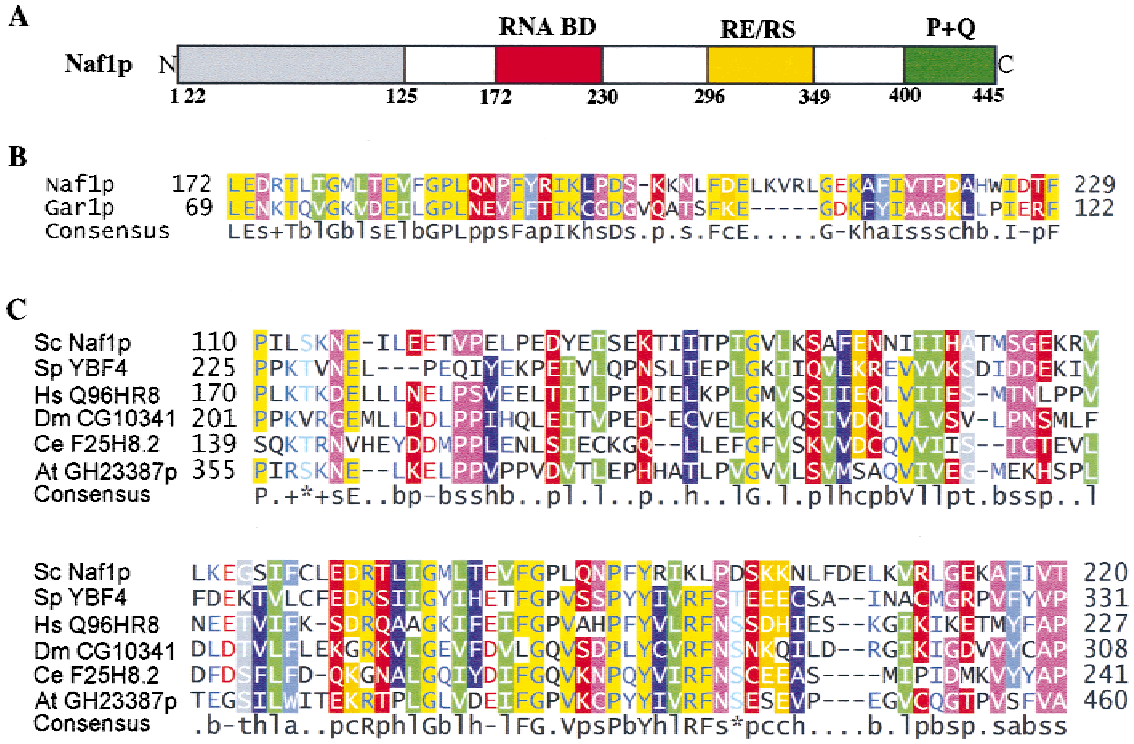

FIGURE 1. A: Schematic representation of Naf1p. Colored boxes indicate regions of homologies with other proteins. Putative domains are indicated. **B:** Alignment of *S. cerevisiae* Naf1p with the RNA-binding domain of yeast Gar1p. **C:** Alignments of Naf1p homologs. SwissProt protein names are shown together with species abbreviations (Sc: *Saccharomyces cerevisiae*; Sp: *Schizosaccharomyces pombe*; Hs: *Homo sapiens*; Dm: *Drosophila melanogaster*; Ce: *Caenorhabditis elegans*; At: *Arabidopsis thaliana*). Numbers flanking the alignments indicate the residue numbers of aligned sequences. Identical residues are shaded yellow and indicated with uppercase letters in the consensus sequences. The lowercase letters on the consensus line have the following meaning: a, aromatic (FHWY); b, big (EFHIKLMQRWY); c, charged (DEHKR); h, hydrophobic (ACFGHILMTVWY); l, aliphatic (ILV); p, polar (CDEHKNQRST); s, small (ACDGNPSTV); +, positively charged (HKR); −, negatively charged (DE).

homology with Gar1p; for example, the *Drosophila* ortholog of Naf1p (CG10341) identified Gar1p in the first iteration ($E = 0.002$) and retrieved other homologs of Gar1p in subsequent iterations (data not shown). The region of sequence similarity is longer than that shown in Figure 1B, although it is discontinuous because of insertions and deletions. Moreover, the two proteins show similarity at the level of secondary structure even when clear primary sequence similarity is absent, and their relatedness is unquestionably solid based on PSI-BLAST statistics. Finally, subsequent TBLASTN searches of the EST database revealed that Naf1p orthologs are widespread in animal and plant species.

Despite being studied experimentally for more than a decade, it is not clear whether Gar1p and its homologs are related to structurally characterized proteins. To address this issue, we performed threading of Naf1p and Gar1p versus a library of known protein structures. Threading is the process where a protein of unknown three-dimensional structure is compared with known folds in an attempt to recognize the structural similarity between them (Bowie et al., 1991; Jones et al., 1992). The compatibility between the sequence and the proposed structure is evaluated by means of a set of empirical potentials derived from proteins of known structure. To improve the chance of finding a correct fold, the original threading protocol (Jones, 1999) was supplemented by secondary structure predictions (Jones, 1999). Both Naf1p and Gar1p showed structural homology to the common fold of the Sm proteins (Kambach et al., 1999) and to the structurally related tudor domain of the SMN protein (Selenko et al., 2001; see Materials and Methods for details). This fold assignment was confirmed by building a robust three-dimensional model of Gar1p using human Sm proteins as a template (M. Dlakić, A. Fatica, & D. Tollervey, in prep.).

Sequence analysis and structural prediction identified second and third globular domains of Naf1p (residues 277–365 and 391–448) that contain a short RE/RS-rich region and a C-terminal P + Q-rich domain, respectively. These are features present in several yeast hnRNP-like proteins (Wilson et al., 1994), including Nrd1p and Nab3p, although the putative RNA-binding domains of these proteins are not homologous to Naf1p (data not shown).

Together these features suggested a role for Naf1p in the metabolism of box H/ACA snoRNAs.

In addition, the N terminus of Naf1p was reported to show 26% identity (44% similarity) to the human protein TFIIA alpha/beta-like factor (ALF; see www.incyte.com/proteome for alignment), a testis-specific homolog of the large subunit of the general transcription factor TFIIA (Upadhyaya et al., 1999; reviewed in Veenstra & Wolffe, 2001). ALF aids the association of the TATA-binding protein (TBP) with promoter regions, and the homology suggested the possibility of interactions between Naf1p and the transcription machinery.

Naf1p is localized to the nucleus

To determine the subcellular localization of Naf1p, a C-terminal fusion between Naf1p and the tandem affinity purification (TAP) tag was constructed and expressed under the control of its own promoter (Naf1-TAP; see Materials and Methods). The fusion protein is the only source of Naf1p in this strain, which had a growth rate identical to that of the otherwise isogenic wild-type strain (data not shown), showing the tagged protein to be fully functional. The protein A moiety of the TAP tag was used to localize the fusion protein by indirect immunofluorescence microscopy with anti-protein A antibodies (Fig. 2C). To allow visualization of the nucleolus, the Naf1-TAP strain was additionally transformed with a plasmid expressing DsRed-tagged Nop1p (Fig. 2B; Milkereit et al., 2001). This decorated a crescent-shaped region of the nucleus, which is characteristic of the yeast nucleolus. The cells were also treated with DAPI to visualize the nucleoplasm (Fig. 2A,F). Superimposition of the different signals (Fig. 2B,D,E) showed that the Naf1-TAP signal overlapped the regions decorated by both DAPI and DsRed-Nop1p. The otherwise isogenic wild-type strain, which was utilized as control, gave no clear signal in the channels used to detect DsRed-Nop1p or Naf1-TAP (Fig. 2F–L). These data indicate that Naf1p is localized to both the nucleolus and nucleoplasm.

Construction of a conditional NAF1 allele

To study the function of Naf1p, we constructed a conditional allele by placing the expression of a N-terminal ProtA-tagged Naf1p under the control of a repressible *GAL10* promoter (strain YAF9). In galactose liquid media, growth of the *GAL::ProtA-naf1* strain was identical to that of the isogenic wild-type strain (data not shown). Following transfer to glucose liquid media, the growth rate of both strains was initially the same, but growth of the *GAL::ProtA-naf1* strain decreased progressively, commencing 15 h after transfer (Fig. 3A). By 24 h after transfer, growth was severely reduced. Western blot analysis confirmed the depletion of ProtA-Naf1p on glucose medium (Fig. 3B).

Naf1p is required for the synthesis of H/ACA snoRNAs

The homology between Naf1p and the RNA-binding domain of Gar1p suggested the possible involvement of Naf1p in the metabolism of box H/ACA snoRNAs, the levels of which were therefore determined during Naf1p depletion (Fig. 4A). Northern hybridization with probes specific for snoRNAs showed that all tested

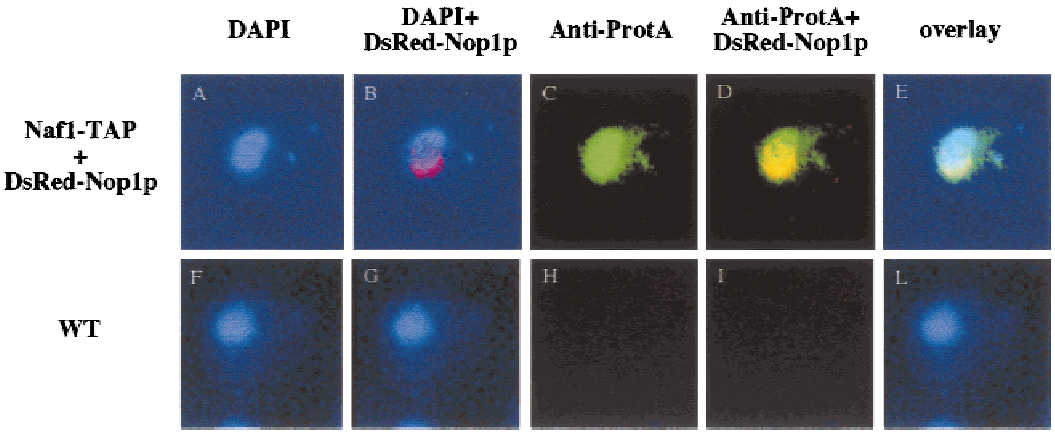

FIGURE 2. Naf1p is localized to the nucleus. Indirect immunofluorescence was performed with cells expressing a TAP-tagged Naf1p and transformed with a plasmid expressing a DsRed-tagged a Nop1p (YAF15 strain). Isogenic wild-type cells were utilized as control. **A,F:** DNA was stained with DAPI. **B,G:** Localization of the DsRed-Nop1p. **C,H:** Indirect immunofluorescence with a rabbit anti-Protein A antibody. **D,I:** Superimposition of signals obtained from Nop1p and Naf1-TAP. **E,L:** Superimposition of the signals obtained from Nop1p, Naf1p, and the DAPI staining.

H/ACA snoRNAs, both independently transcribed and intron encoded, were strongly depleted following transfer of the *GAL::ProtA-naf1* strain to glucose medium. In contrast, no depletion was observed for any tested box C/D snoRNAs, the U4 snRNA, or the RNA component of the signal recognition particle, scR1. The signals for these RNAs actually increased during depletion of Naf1p. It should, however, be noted that the gels were loaded using constant amounts of total RNA, and depletion of the rRNAs (see below) leads to some overloading of RNAs that are not depleted (Henras et al., 1998; Lafontaine et al., 1998).

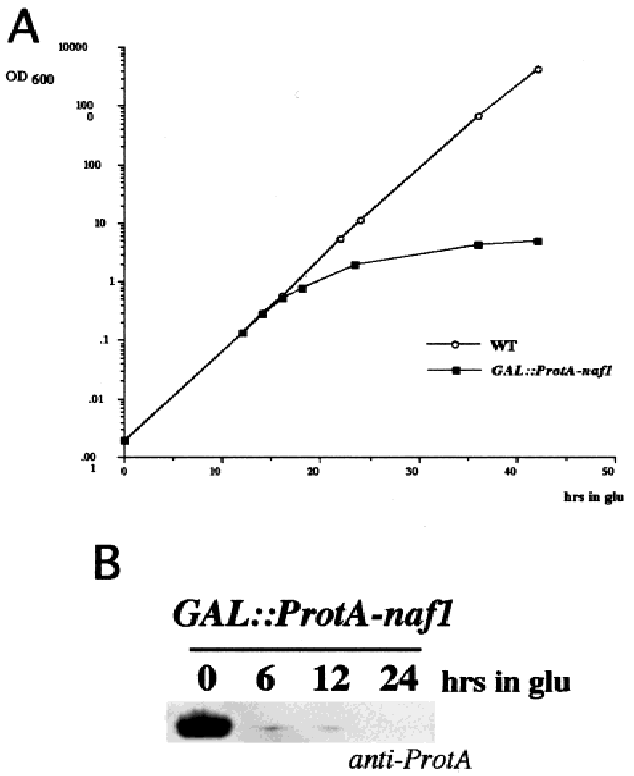

FIGURE 3. A: Growth rate of GAL::ProtA-naf1 (squares) and wild-type (circles) strains following a shift from galactose- to glucose-containing media. **B:** Western blot analysis of Naf1p depletion. Whole-cell extracts were prepared from samples harvested at the indicated times. Equal amount of proteins were separated by 12% SDS-PAGE and ProtA-Naf1p was detected by western blot using peroxidase-conjugated rabbit IgG (Sigma).

To investigate the association of Naf1p with the box H/ACA snoRNAs, immunoprecipitation was performed on lysates of the Naf1-TAP strain and an isogenic strain expressing only nontagged Naf1p (Fig. 4B). No significant coprecipitation was observed for most of the tested independently transcribed H/ACA snoRNAs and the box C/D snoRNA U3. Stronger coprecipitation was seen for snR30 and snR44, the only intron-encoded box H/ACA snoRNA in yeast. This may be specific, as similar levels of recovery were observed when precipitation was performed in 150 or 500 mM NaCl. Recovery of weakly associated RNAs is expected to be reduced under the more stringent conditions of 500 mM NaCl. We conclude that Naf1p is not a major component of the box H/ACA snoRNPs but may transiently associate with the snoRNAs. The observation that Naf1-TAP did not show nucleolar enrichment also suggested that it is not associated with most of the H/ACA snoRNA population. We therefore predicted that Naf1p was specifically required for the synthesis of H/ACA snoRNAs or RNP assembly.

Naf1p is required for pre-rRNA processing

To test the requirement for Naf1p in rRNA synthesis, the levels of mature rRNA and pre-rRNAs were assessed by northern hybridization during Naf1p depletion (Fig. 5). RNA was extracted from the *GAL::ProtA-naf1* and wild-type strains grown in galactose medium

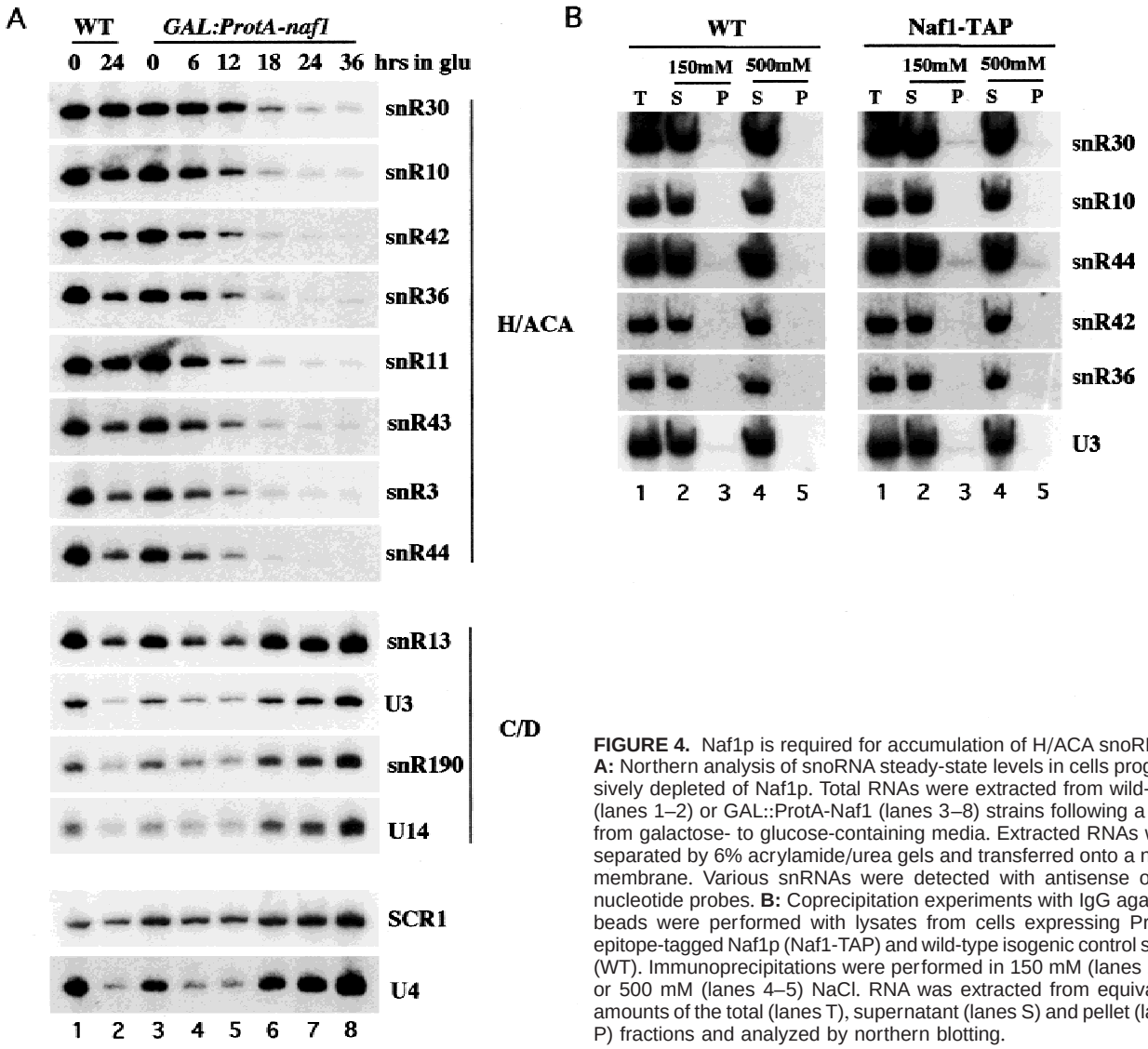

FIGURE 4. Naf1p is required for accumulation of H/ACA snoRNAs. **A:** Northern analysis of snoRNA steady-state levels in cells progressively depleted of Naf1p. Total RNAs were extracted from wild-type (lanes 1–2) or GAL::ProtA-Naf1 (lanes 3–8) strains following a shift from galactose- to glucose-containing media. Extracted RNAs were separated by 6% acrylamide/urea gels and transferred onto a nylon membrane. Various snRNAs were detected with antisense oligonucleotide probes. **B:** Coprecipitation experiments with IgG agarose beads were performed with lysates from cells expressing Prot A epitope-tagged Naf1p (Naf1-TAP) and wild-type isogenic control strain (WT). Immunoprecipitations were performed in 150 mM (lanes 2–3) or 500 mM (lanes 4–5) NaCl. RNA was extracted from equivalent amounts of the total (lanes T), supernatant (lanes S) and pellet (lanes P) fractions and analyzed by northern blotting.

or shifted to glucose medium and resolved on agarose/formaldehyde gels (Fig. 5D–I) or acrylamide/urea gels (Fig. 5J–L). Following transfer of the *GAL::ProtA-naf1* strain to glucose medium, the mature 18S rRNA was depleted (Fig. 5I) as were the $27SA_2$ (Fig. 5D–F) and 20S (Fig. 5H) pre-rRNAs. In contrast, the 35S pre-rRNA was strongly accumulated (Fig. 5D) and the aberrant 23S RNA appeared (Fig. 5D,E,H). The 23S RNA originates from direct cleavage of the 35S pre-rRNA at site A_3 in the absence of prior cleavage at sites A_0, A_1, and A_2 (see Fig. 5C). Much less depletion was seen for the 27SB pre-rRNA (Fig. 5F) or 25S rRNA (Fig. 5G) in the *GAL::ProtA-naf1* strain on glucose medium. A further aberrant RNA was accumulated following Naf1p deletion, which is predicted to extend from site D, the 3′ end of the 18S rRNA, to site B_2, the 3′ end of the 25S rRNA (labeled D-B_2 in Fig. 5D–F). This RNA was detected with probes 001, 003, 006 (Fig. 5), and 002 (data not shown).

Northern analysis of low molecular weight RNAs showed that later precursors on the 5.8S synthesis pathway, the 7S and 6S pre-rRNAs, were not depleted (Fig. 5J), and the mature 5.8S was only mildly depleted in the *GAL::ProtA-naf1* strain on glucose medium (Fig. 5H–L). The increased signal for the 7S and 6S pre-rRNAs and the 5S RNA (Fig. 5L) may be a consequence of the depletion of the 18S rRNA, leading to increased loading of other RNA species, or may reflect a mild delay in processing. The increased signal seen in the wild-type strain between the 0 h (galactose) and the 24 h (glucose) samples is a consequence of nutritional upshift increasing ribosome synthesis.

The pre-rRNA defects seen in the *GAL::naf1* strain on glucose medium reveal an inhibition of the early pre-rRNA cleavages at sites A_0, A_1, and A_2 (see Fig. 5B,C). Similar defects were observed following depletion of the box H/ACA snoRNAs snR10 and snR30 (Tollervey, 1987; Morrissey & Tollervey, 1993). The loss

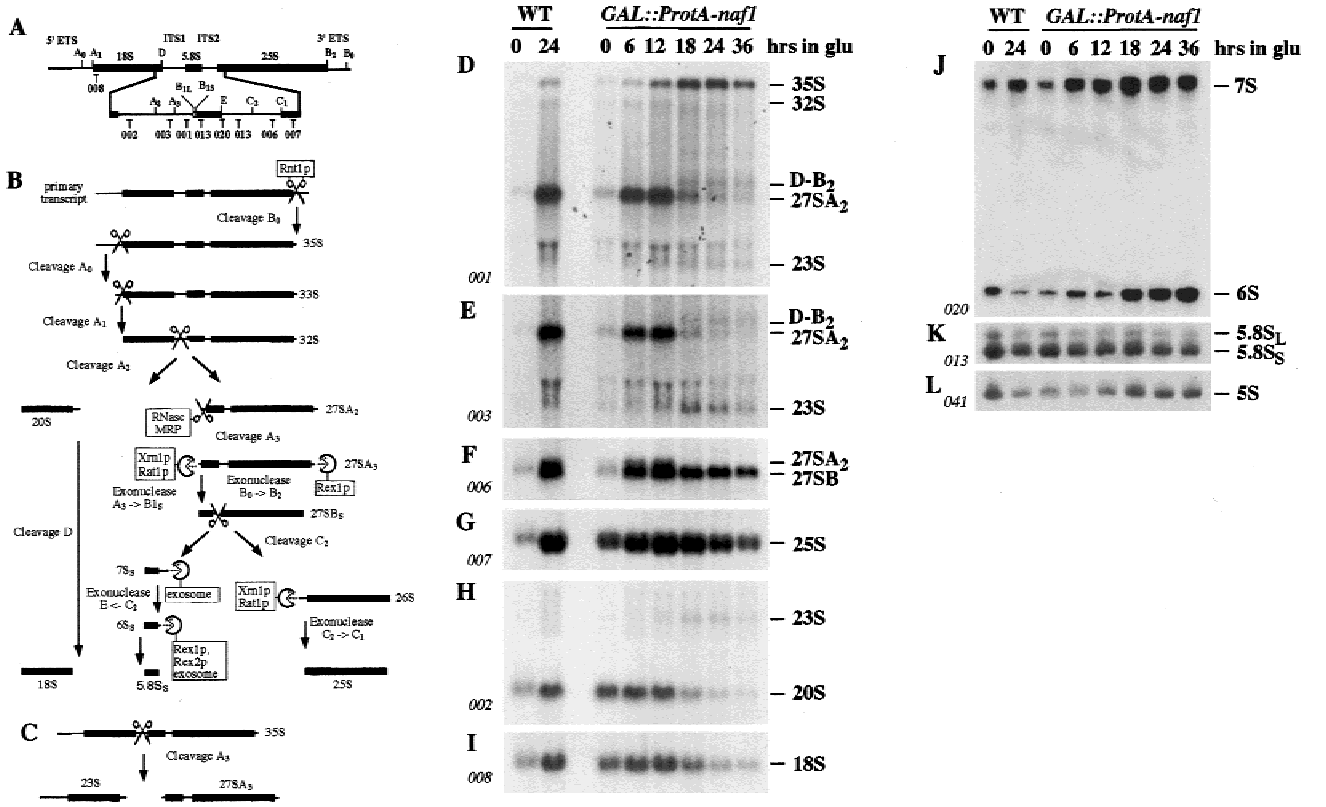

FIGURE 5. Naf1p is required for pre-rRNA processing. **A:** Structure and processing sites of the 35S pre-rRNA. This precursor contains the sequences for the mature 18S, 5.8S, and 25S, which are separated by the two internal transcribed spacers, ITS1 and ITS2, and flanked by the two external transcribed spacers, 5′ETS and 3′ETS. The positions of the oligonucleotides probes utilized in northern hybridizations are indicated. **B:** Pre-rRNA processing pathways. In wild-type cells, the 35S pre-rRNA is first cleaved at site A_0, producing the 33S pre-rRNA; this molecule is rapidly cleaved at site A_1 to produce 32S pre-rRNA. 32S is cleaved at site A_2, releasing 20S and $27SA_2$. $27SA_2$ is then processed via two alternative pathways. It may be cut at site A_3 to produce $27SA_3$, which is then trimmed to site B_{1S}, producing $27SB_S$. Alternatively, it can be processed to $27SB_L$ by an as yet unknown mechanism. $27SB_S$ and $27SB_L$ are matured to the 5.8S and 25S rRNAs following identical pathways. Cleavage at site C_2 generates the 7S and 26S pre-rRNAs. The 7S pre-rRNA is digested 3′ to 5′ to 6S pre-rRNA and then to the mature 5.8S rRNA. The 26S pre-rRNA is digested 5′ to 3′ to the 25S′ pre-rRNA and then to the mature 25S rRNA. For a review on pre-rRNA processing and the known processing enzymes see Venema and Tollervey (1999) and Kressler et al. (1999). **C:** Structure of aberrant pre-rRNA processing intermediate 23S, which is generated from premature cleavage in the 35S pre-rRNA at site A_3 in the absence of cleavage at sites A_0, A_1, and A_2. **D**–**L:** Northern analysis of pre-rRNA processing. Strains *GAL-ProtA-naf1* and wild-type were grown at 30 °C in YPGal then shifted to YPD. Cells were harvested at the indicated times and total RNAs were extracted. Equal amounts of RNA (5 μg) were resolved on 1.2% agarose/formaldehyde gels (**D**–**I**) and 6% acrylamide/urea gels (**J**–**L**) and transferred to a nylon membrane. The membranes were consecutively hybridized with the probes indicated in **A**. The position of the mature rRNAs and pre-rRNAs are indicated.

of snR10 and snR30 from the *GAL::ProtA-naf1* strain on glucose medium may underlie these processing defects. However, the appearance of the D-B_2 fragment was not seen on depletion of snR10 or snR30, and has not been reported for strains lacking other box H/ACA snoRNAs or snoRNP proteins, but may be a consequence of the global loss of the H/ACA snoRNAs.

Naf1p is not required for specific transcription of H/ACA snoRNAs

The homology between Naf1p and the human transcription factor ALF suggested that it might function as a transcription factor specific for the promoters of independently transcribed H/ACA snoRNA genes and the host gene for snR44.

To test this, a one-step PCR technique was used to replace the promoter of the chromosomal *SNR10* gene with the regulated *MET3* promoter (Colley et al., 2000) in the *GAL::ProtA-naf1* strain. Correct integration was confirmed by PCR and expression of snR10 was shown to be subject to methionine repression (Fig. 6A, compare lanes 3 and 4). The abundance of snR10 in galactose medium was slightly lower in the *MET::snr10*; *GAL::ProtA-naf1* strain than in the wild type, very likely due to lower efficiency of the *MET3* promoter compared to snoRNA promoters. Even a strong *GAL*-promoter underexpressed snR30 (Morrissey & Tollervey, 1993). The *MET::snr10*; *GAL::ProtA-naf1* and wild-type strains were grown without methionione, to induce snR10 expression, in galactose medium, and transferred to glucose medium to repress Naf1p synthesis.

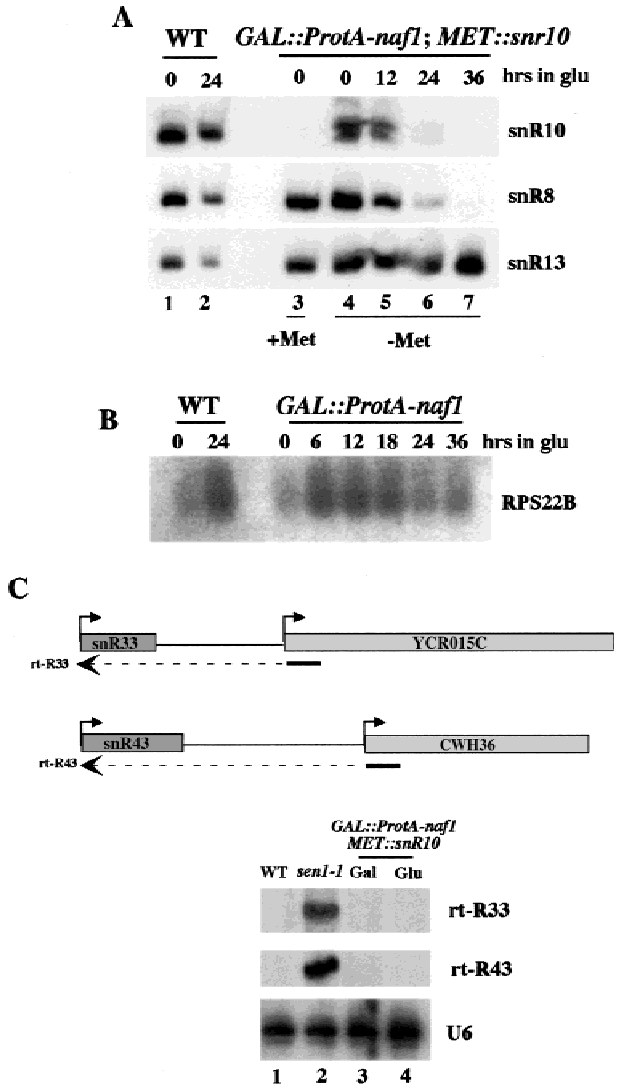

FIGURE 6. Naf1p is not specifically required for transcription or 3′-end formation on H/ACA snoRNA genes. **A:** Northern analysis of snoRNA steady-state levels in wild-type (lanes 1–2) and *GAL::ProtA-naf1; MET::snR10* (lanes 3–7) strains. Cells were grown in galactose medium and shifted to glucose medium for the time indicated. Total RNA was extracted from cells grown in selective minimal medium lacking methionine with the exception of lane 3, in which methionine was added to a final concentration of 20 mM. RNAs were resolved on 6% acrylamide/urea gel, transferred to a nylon membrane, and hybridized with oligonucleotides specific for the H/ACA snoRNAs snR10 and snR8, and the box C/D snoRNA snR13. **B:** Accumulation of RPS22B mRNA in Naf1p-depleted cells. The membrane utilized for the hybridization is the same as in Figure 5. **C:** Oligonucleotides complementary to the coding strand of the YCR015C and CWH36 genes were utilized for a primer extension analysis on total RNA extracted from wild-type (lane 1), *sen1-1* (lane 2), and *GAL::ProtA-naf1* (lanes 3–4) strains. The *sen1-1* strain were grown at 25 °C and then shifted to 37 °C for 1 h. *GAL::ProtA- naf1* strain was grown in galactose medium and shifted to glucose medium for 24 h. The U6 snRNA was utilized as a normalization control. As schematically represented above the gels, the extended products correspond to an elongated RNA starting at the 5′ end of the snoRNA transcripts.

Northern hybridization showed that the H/ACA snoRNAs snR10 and snR8 were strongly, and apparently equally, reduced on depletion of Naf1p, whereas no depletion was seen for the box C/D snoRNA snR13 (Fig. 6A, lanes 4 to 7). Accumulation of snR10 was therefore dependent on Naf1p even when a heterologous regulated promoter drove its transcription.

The host gene for snR44 is *RPS22B* and the level of the mRNA transcribed from this gene was also assessed by northern hybridization during Naf1p depletion (Fig. 6B). The level of the *RPS22B* mRNA strain showed the effects of nutritional upshift following transfer to glucose medium, as expected for a ribosomal protein gene, and then decreased only slightly even after 24 h in glucose, by which time growth was severely reduced (Fig. 6A and Fig. 3A). In contrast, the levels of snR44 were decreased 6 h after transfer and were strongly reduced after 12 h in glucose (see Fig. 4).

From these results, we conclude that Naf1p acts after transcription initiation.

Naf1p is not required for termination of snoRNA transcripts

Naf1p shares structural features with Nrd1p, a protein implicated in the synthesis of both snRNAs and snoRNAs (Steinmetz et al., 2001). Strains carrying the *nrd1-5*, *sen1-1*, or *rna15-2* mutations are defective in 3′-end formation and transcription termination on snRNA and snoRNA genes, leading to the appearance of read-through transcripts (Steinmetz et al., 2001; Morlando et al., 2002). We therefore tested for the presence of H/ACA snoRNA read-through transcription products in the *GAL::ProtA-naf1* strain (Fig. 6C). Primer extension analysis was performed with primers complementary to the mRNAs YCR015C and CWH36, which are transcribed from genes that lie immediately downstream of SNR33 and SNR43, respectively (see schematic representation in Fig. 6C). As positive control, primer extension analyses were performed on RNA extracted from a *sen1-1* strain 1 h after transfer to 37 °C (Fig. 6C, lane 2), in which read-through transcripts were readily detected (Steinmetz et al., 2001). No read-through transcripts were detected in the wild-type strain (Fig. 6C, lane 1) or in the *GAL::ProtA-naf1* strain either grown in galactose medium or 24 h after transfer to glucose (Fig. 6C, lanes 3–4). The pol III-transcribed U6 snRNA was used as a normalization control. We conclude that Naf1p is not required for transcription termination on H/ACA snoRNA genes.

Naf1p binds the H/ACA snoRNP proteins Nhp2p and Cbf5p

During the course of this work, interactions between Naf1p and two H/ACA snoRNP proteins were reported from high-throughput screens; Naf1p was reported to interact with Cbf5p and Nhp2p in a two-hybrid screen (Ito et al., 2001) and was copurified with overexpressed FLAG-tagged Cbf5p in a proteomic analysis (Ho et al., 2002). To confirm these putative interactions and de-

termine whether they are mediated by direct contacts, coprecipitation assays were performed in vitro (Fig. 7A). A bacterially expressed GST–Naf1p fusion protein was immobilized on glutathione beads. These were then incubated with in vitro translated, [^{35}S]methionine labeled Nhp2p and Cbf5p (Fig. 7A, lane 1). GST-Naf1p specifically retains Nhp2p and Cbf5p (Fig. 7A, lane 3) whereas no retention was observed using GST alone (Fig. 7A, lane 2). This result confirms the reported in vivo interactions, and shows that they are not dependent on intermediary proteins.

Naf1p is an RNA-binding protein

The potential RNA-binding activity of Naf1p was assessed by bandshift analyses using recombinant GST-tagged Naf1p and [^{32}P]-labeled in vitro-transcribed snR36 (Fig. 7B). Several retarded complexes were obtained with GST-Naf1p (Fig. 7B, lanes 2–4) but not with GST alone (Fig. 7B, lane 5), indicating that the protein can bind directly to RNA. The multiple complexes formed between Naf1p and snR36 suggests that in our experimental condition, the protein can interact with several binding sites in the snoRNA. To investigate the specificity of the RNA binding, we carried out competition experiments using a 200-fold molar excess of unlabeled RNAs (Fig. 7C). Both snR36 and its antisense transcript were able to efficiently compete for the binding (Fig. 7C, lanes 3–5). In contrast, no competition was observed with the snRNA U6 and tRNAs (Fig. 7C, lanes 4–6). These data indicate that GST-Naf1p probably recognizes structural features more than specific sequences during RNA binding in vitro. Similar observations have been made for the box H/ACA protein Nhp2p,

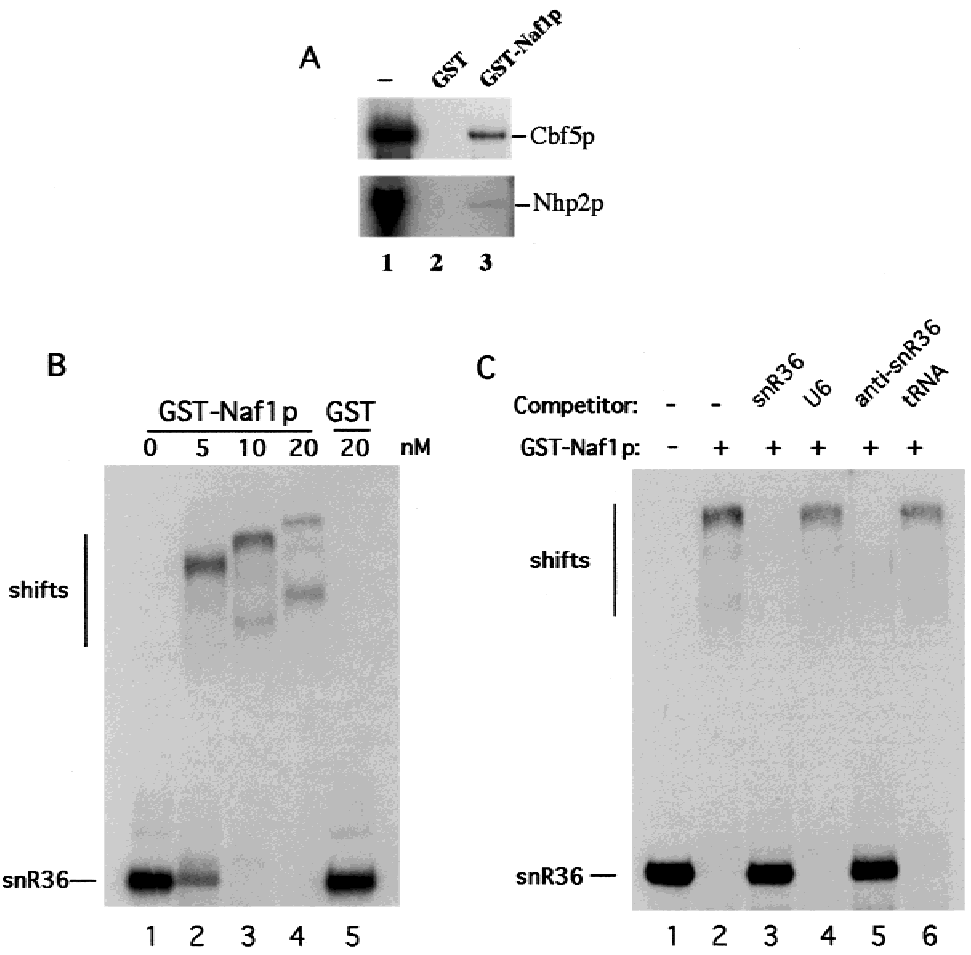

FIGURE 7. Naf1p physically interacts with Cbf5p, Nhp2p, and RNA. **A:** In vitro-translated ^{35}S-labeled Cbf5p and Nhp2p (lane 1) were individually utilized in GST pull-down experiments with recombinant GST (lane 2) or GST-Naf1p protein (lane 3). Proteins recovered from each experiment were analyzed by SDS-PAGE. **B:** Gel mobility shift assay performed with the in vitro-transcribed H/ACA snoRNA snR36. GST-Naf1p or GST purified from *E. coli* were used at the concentration indicated. **C:** Competition experiment. Gel shift assay was performed in the presence of 200-fold molar excess of the following competitor RNAs: snR36 (lane 3), U6 snRNA (lane 4), antisense RNA of snR36 (lane 5), tRNA (lane 6). Reactions in lanes 2–6 contained GST-Naf1p at a concentration of 10 mM. Complexes were resolved by electrophoresis on native 6% acrylamide/bisacrylamide (80:1) gels.

which binds to imperfect stem-loop structures in vitro, rather than to specific sequences (Henras et al., 2001).

Naf1p binds to the CTD of RNA polymerase II

Many hnRNP-like proteins, including Nrd1p, bind to the C-terminal domain of the largest subunit of RNA polymerase II. Two-hybrid screens have previously been used to assess the interactions between the CTD and RNA processing factors including Rna15p, Pcf11p, and Nrd1p (Yuryev et al., 1996; Barilla et al., 2001). The potential in vivo association of Naf1p and the CTD was assessed using a yeast two-hybrid system previously used to show the interaction of the cleavage and polyadenylation factors Pcf11p and Yhh1p with the CTD (Fig. 8A; Barilla et al., 2001; B. Dichtl & W. Keller, pers. comm.). The CTD of the yeast RNA polymerase II was expressed as a fusion with the *GAL4* DNA-binding domain, whereas the full-length Naf1p sequence was expressed as a fusion with the transcription activation domain (see Materials and Methods). Interaction between these fusion proteins activates transcription of both the *lacZ* and *HIS3* genes. Coexpression of the CTD and Naf1p-fusion constructs robustly stimulated expression of β-galactosidase as judged by a filter color development assay (data not shown) or in enzymatic activity in cell extracts (Fig. 8A) compared to control strains expressing the constructs individually. To test for expression of *HIS3*, we assessed the ability of the strains to grow in the absence of exogenous histidine. Substantially better growth was seen for the strain expressing both the binding domain-CTD fusion and the activation domain Naf1p fusion than for the control strains lacking either fusion construct (data not shown).

To confirm this interaction, we performed a GST-CTD pull-down experiment on cell extracts from the strain expressing TAP-tagged Naf1p (Fig. 8B). Glutathione affinity beads were prepared containing immobilized GST (Fig. 8B, lanes 2 and 5), nonphosphorylated GST-CTD (Fig. 8, lanes 3 and 6), or phosphorylated GST-CTD (Fig. 8, lanes 4 and 7). Yeast whole extract was incubated with the beads and, after extensive washing, the bound material was recovered and analyzed by western blotting. Anti-ProtA antibodies were utilized to detect Naf1-TAP and anti-Nop1p antibodies to detect Nop1p, as negative control. Naf1-TAP was retained on the beads carrying the phosphorylated CTD, but was not retained by the nonphosphorylated CTD or the control GST. The efficiency of Naf1p precipitation was low, but comparable to that reported for Pcf11p, which other assays show to interact with the CTD (Barilla et al., 2001).

The yeast two-hybrid analysis and the GST pull-down from cell extracts do not discriminate between a direct association and indirect interactions, involving one or more partners. In vitro-translated Naf1p was therefore tested for its association with the GST-CTD. Under these conditions, Naf1p did not show clear association with the phosphorylated or nonphosphorylated CTD (data not shown). Similar behavior has been reported for Rna15p (Barilla et al., 2001), and this suggests that the interaction between CTD and Naf1p may be indirect.

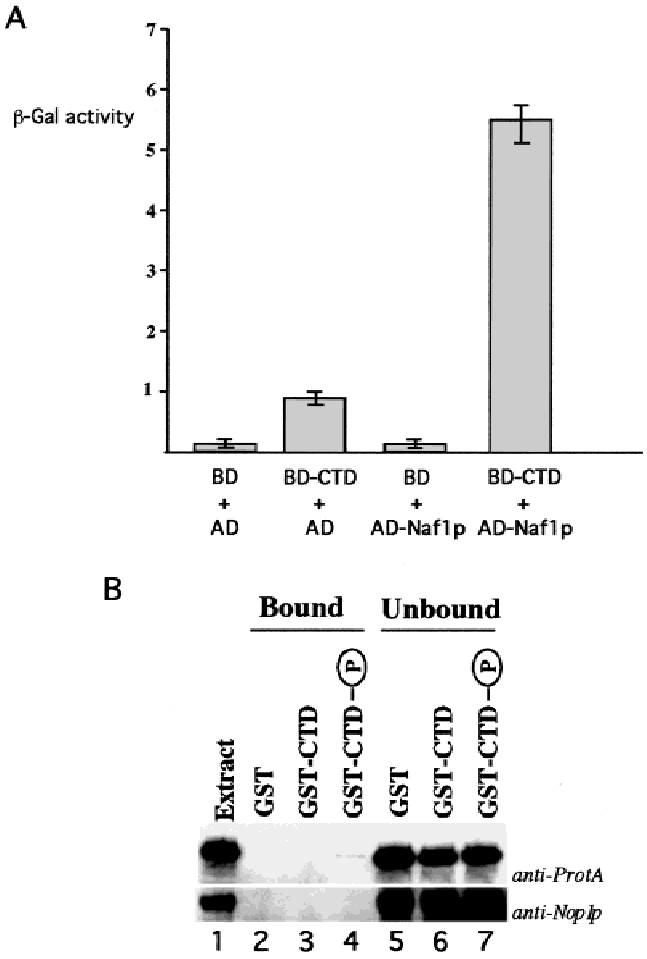

FIGURE 8. A: Quantitative liquid β-galactosidase assays for the two-hybrid interaction between the CTD and Naf1p. Mean values are shown in the graph. **B:** Naf1p associates with phosphorylated CTD. Whole-cell extract from the Naf1-TAP strain was utilized in GST pull-down experiments with GST (lanes 2, 5), GST-CTD (lanes 3, 6), and phosphorylated GST-CTD (lanes 4, 7). The yeast extract (2% of total input; lane 1), bound fractions (100%, lanes 2–4), and supernatant fractions (10%; lanes 5–7) were loaded onto a SDS-PAGE and analyzed by western blot with rabbit anti-Protein A antibody and a mouse monoclonal anti-Nop1p.

DISCUSSION

Based on sequence homology with the H/ACA snoRNP protein Gar1p, we identified a novel essential protein that we termed Naf1p. The results presented in this study show that Naf1p is required for a posttranscriptional step in box H/ACA snoRNP synthesis. Naf1p is not a component of the major box H/ACA snoRNP

population but is required for the accumulation of all tested box H/ACA snoRNAs. Four proteins, Gar1p, Cbf5p, Nhp2p, and Nop10p, are stable components of the box H/ACA snoRNPs (Kiss, 2002). However, although Cbf5p, Nhp2p, and Nop10p are each required for box H/ACA snoRNA accumulation, Gar1p is dispensable for snoRNA accumulation (Girard et al., 1992; Henras et al., 1998; Lafontaine et al., 1998). The H/ACA snoRNP proteins are reported to associate with the snoRNA precursors in strains in which processing is blocked (Henras et al., 1998) suggesting they, and Naf1p, bind to the snoRNAs at an early stage of H/ACA snoRNP assembly, possibly during transcription.

Naf1p-related proteins are widespread in eukaryotes, indicating that they perform a conserved function. The family contains a region that is clearly related to the RNA-binding domain of yeast Gar1p and its homologs. The functional significance of this homology is supported by the in vitro RNA-binding activity of Naf1p. During the course of these analyses, Naf1p was reported to interact with the box H/ACA snoRNP proteins Cbf5p and Nhp2p in a high-throughput two-hybrid screen (Ito et al., 2001), and was copurified with overexpressed FLAG-tagged Cbf5p in a proteomic analysis (Ho et al., 2002). Here we show that Naf1p binds directly Cbf5p and Nhp2p in vitro, and also interacts with the CTD of RNA pol II. We therefore propose that Naf1p associates with the CTD of RNA pol II and binds to newly synthesized or nascent box H/ACA snoRNAs. Naf1p then either recruits Nhp2p and Cbf5p to the snoRNP or binds together with them as a preformed complex. The low specificity observed for in vitro RNA binding with Naf1p alone and the fact that coprecipitation was not detected between the CTD and recombinant Naf1p alone suggests that the latter may be the case.

In view of the sequence and predicted structural homology between Naf1p and the RNA-binding domain of Gar1p, we propose that they bind to the same regions of the snoRNAs. Once the snoRNP structure is established, Gar1p may replace Naf1p, as the former is a major component of the mature snoRNPs, unlike Naf1p itself. It may well be that displacement of Naf1p requires the completion of later snoRNA processing events. These may involve the putative ATPase p50/Rvb2p that has been implicated in the formation of both C/D and H/ACA snoRNPs, and depletion of which results in delocalization of Gar1p (King et al., 2001). In the absence of correct initial assembly with Naf1p and the snoRNP proteins, the snoRNAs are presumably rapidly degraded. The activity responsible for this degradation has not been determined, but the exosome complex, which degrades other defective nuclear RNA precursors (Bousquet-Antonelli et al., 2000; Torchet et al., 2002), appears a likely candidate.

It is notable that there are other known precursor-specific factors with homology to RNA-binding proteins that are present in mature RNPs. Rlp7p and Rlp24p, which are homologous to ribosomal proteins Rpl7p and Rpl24p, respectively, are present in pre-60S ribosomal particles but absent from mature ribosomes (Dunbar et al., 2000; Saveanu et al., 2001).

Human H/ACA snoRNPs can be assembled in vitro (Dragon et al., 2000), but the in vivo assembly pathway is likely to involve additional factors (Terns & Terns, 2001). In human cells, and probably most other eukaryotes, assembly of small nuclear and small nucleolar RNP complexes is likely to be mediated by the SMN complex (reviewed in Terns & Terns, 2001). *S. cerevisiae* lacks clear homologs of the SMN complex components, but we propose that Naf1p fulfils a function in box H/ACA snoRNP assembly that is in some way analogous. The region conserved between Naf1p and Gar1p has a predicted fold that is similar to that of the tudor domain of SMN (Selenko et al., 2001) and the conserved core of the Sm proteins (Kambach et al., 1999). The tudor domain of human SMN binds a repeat domain rich in glycine and dimethylated arginine (GAR or RGG domain) that is present in Gar1p as well as the box C/D snoRNP protein fibrillarin and the Sm protein components of the spliceosomal snRNPs (Fischer et al., 1997; Buhler et al., 1999; Friesen & Dreyfuss, 2000; Brahms et al., 2001; Jones et al., 2001; Pellizzoni et al., 2001a; Selenko et al., 2001). In the case of the Sm proteins, this interaction predominately involves the recognition of symmetrical arginine dimethylation (sDMA; Brahms et al., 2001). Whether yeast proteins contain sDMA has not been determined, but *S. cerevisiae* Hsl7p (Ybr133p) is a functional homolog of human JPB1, the methyltransferase component of the 20S methylosome complex that is responsible for this modification (Lee et al., 2000; Friesen et al., 2001; Meister et al., 2001). Additionally, in the nucleus, the SMN complex is physically and functionally associated with RNA pol II (Pellizzoni et al., 2001b), and SMN can bind directly to the U1 snRNA, an activity that is required for snRNP assembly (Yong et al., 2002).

There are, therefore, some apparent similarities between Naf1p and SMN; both bind, directly or indirectly, to RNA polymerase II, have a region with an Sm fold, and are implicated in box H/ACA snoRNP assembly. However, humans and other eukaryotes have a protein that is more closely related to Naf1p and more likely to be a direct homolog. Whether and how hNaf1p and SMN interact during snoRNP assembly remains to be determined.

MATERIALS AND METHODS

Strains and microbiological techniques

Standard techniques were employed for growth and handling of yeast. Yeast strains used in this work are: CYM1333 (a, trp1-Δ, his3-Δ, ura3-52, lys2-801, ade2-101), YAF9 (a, trp1-Δ, his3-Δ, ura3-52, lys2-801, ade2-101, HIS3::GAL::ProtA-Naf1),

YAF10 (a, ade2, his3, leu2, trp1, ura3, NAF1::TAP::TRP1), YAF15 (a, ade2, his3, leu2, trp1,ura3, NAF1::TAP::TRP1 [pUN100 Dsred-Nop1, LEU2]), YAF25 (a, trp1-Δ his3-Δ, ura3-52,lys2-801,ade2-101, HIS3::GAL::ProtA-Naf1,TRP1 ::pMET::snR10), and Y190 (MATa ura3-52, trp1-901, ade2-101, leu2-3, 112 his3-200r gal4Δ gal80Δ, URA3::GAL1-lacZ, LYS2::GAL1-HIS3 cyhr). The YAF9 and YAF25 strains were created by use of a one-step-PCR strategy as previously described (Lafontaine & Tollervey, 1996; Colley et al., 2000). TAP tagging of Naf1p was performed as described in Rigaut et al. (1999).

Oligonucleotides

Oligonucleotides anti-snR10, anti-snR30, anti-snR41, anti-snR42, anti-snR36, anti-snR11, anti-snR43, anti-snR3, anti-snR44, anti-snR13, anti-snR190, anti-U3, anti-u14, anti-U4, and anti-scR1 were previously described (Chanfreau et al., 1998; Lafontaine & Tollervey, 1999). Oligonucleotides used for pre-rRNAs analysis were described in Fatica et al. (2002). Oligonucleotides utilized for primer extension analysis were: anti-YCR015, CTGAAATAATAATGGTTTTC, anti-CWH36, TACGTTCCTTTGCCAGCTCCAT, and antiU6, AAAACGAA ATAAATTCTTTGTAAAAC.

RNA extraction, northern hybridization, and primer extension

For depletion of the Naf1p protein, cells were harvested at intervals following a shift from RSG medium (2% galactose, 2% sucrose, 2% raffinose), or YPGal medium containing 2% galactose, to YPD medium containing 2% glucose. Otherwise strains were grown in YPD medium. RNA was extracted as described previously (Fatica et al., 2002). Northern hybridization and primer extension were as described (Steinmetz et al., 2001; Fatica et al., 2002). Standard 1.2% agarose/formaldehyde and 6% acrylamide/urea gels were used to analyze the high and low molecular weight RNA species, respectively.

Immunoprecipitation of ProtA epitope-tagged Naf1p

YAF10 and wild-type strains were utilized for the immunoprecipitation experiments. Immunoprecipitations were performed on IgG-agarose beads as previously described (Colley et al., 2000). RNA from the total, supernatant, and pellet fractions were resolved on a 6% acrylamide/urea gel and analyzed by Northern blot analysis.

Fluorescence microscopy

Indirect fluorescence was performed in the YAF10 strain as previously described (Grandi et al., 1993). Plasmid pUN100 DsRed-Nop1 was introduced into yeast cells by transformation, and selected on SD-URA medium. Individual transformants were grown in selective medium, fixed by incubation in 4% (v/v) formaldehyde for 30 min at 25 °C, and spheroplasted. TAP fusion was detected with a rabbit anti-Protein A antibody (Sigma) and a secondary goat anti-rabbit antibody coupled to FITC (Sigma) at a 1:100 and a 1:200 dilution, respectively. To stain nuclear DNA, DAPI was included in the mounting medium (Vectashield, Vector Laboratories). Cells were viewed on a Zeiss Axioscop fluorescence microscope and pictures were obtained with a Xillix Microimager CCD camera.

In vitro protein-binding assay

PCR fragment corresponding to the YNL124W (NAF1) open reading frame (ORF) was amplified by genomic DNA and cloned into the pGEX-2T vector (Pharmacia) to obtain the pGEX-NAF1 vector. NHP2 and CBF5 ORFs were amplified and cloned into the Bluescript KS vector (Stratagene) to generate the BS-NHP2 and BS-CBF5 vectors. The [^{35}S]methionine-labeled proteins were produced by an in vitro coupled transcription–translation reaction (Promega) in the presence of [35S]methionine (Amersham). All the glutathione S-transferase (GST) fusion proteins were expressed from the GST expression vector pGEX-2T (Pharmacia) in the *Escherichia coli* strain BL21 and purified by using glutathione-Sepharose according to the manufacturer's protocol (Pharmacia). GST or GST–Naf1p was incubated with 25 μL bed volume of glutathione–Sepharose beads in 300 μL of NET buffer (20 mM Tris, pH 8, 100 mM NaCl, 0.2 mM PMSF) for 30 min at 4 °C. After extensive washing in 1 M NaCl and then in 100 mM NaCl, [^{35}S]methionine-labeled Nhp2p or Cbf5p was incubated with GST and GST–Naf1p prebound beads. Incubation was allowed to proceed for 30 min at 4 °C. Beads were treated with RNase A and washed four times with NET buffer. Bound proteins were then recovered by heating for 4 min at 95 °C in loading buffer and analyzed by SDS-PAGE. [^{35}S]methionine-labeled proteins were detected by fluorography. GST-CTD was phosphorylated for 2 h at 30 °C with casein kinase I (New England Biolabs) according to the manufacturer's protocol. GST pull-down experiments were performed as described above, but SDS/PAGE gels were transferred to nitrocellulose membranes and analyzed by immunoblotting. TAP-Naf1p and Nop1p were detected with a 1:100 dilution of a rabbit anti-Protein A antibody (Sigma) and a mouse monoclonal anti-Nop1p, respectively. A 1:200 dilution of secondary peroxidase-conjugated antibodies (Sigma) was utilized for the detections.

Mobility shift assays

In vitro transcription of RNAs was performed following standard procedures with templates obtained by genomic PCR amplification and carrying the T7 promoter. The binding reaction was carried out in binding buffer (30 mM Tris-HCl, pH 7.4, 150 mM KCl, 2 mM $MgCl_2$, 0.1% Triton X-100, 20% glycerol) in the presence of 1 mM DTT, tRNA (1 μg/μL), 10 fmol of ^{32}P-labeled RNA, and 0–20 mM of recombinant protein in a reaction volume of 10μL. Labeled RNA was heat denatured at 65 °C for 10 min, followed by slow cooling to room temperature, and then added to the binding reaction. For competition experiments, a 200-fold molar excess of cold competitor RNA was added. The reactions were incubated at room temperature for 30 min and then loaded on a 6% native acrylamide/bisacrylamide (80:1), 4% glycerol gels in 0.5× TBE buffer. Prior to loading, the gels were prerun for 1 h and then run for 3 h at 250 V in the cold room.

Sequence analysis and threading

Initial sequence searches were done on a yeast proteome using FASTA (Pearson & Lipman, 1988) with default parameters. Subsequent PSI-BLAST searches (Altschul et al., 1997) of the nonredundant protein database were done with $E = 0.01$ as the inclusion threshold for further iterations.

Secondary structure predictions were carried out using position-specific scoring matrices obtained from PSI-BLAST searches (Jones, 1999). Predictions for each residue were assigned the confidence on a scale of 0–9, and only predictions with confidence 7 or higher were used for threading. In retrospect, the secondary structure predictions of Gar1p and Naf1p alone were indicative of their potential relatedness to Sm proteins because they share the same pattern: an N-terminal α-helix followed by five β-strands (Kambach et al., 1999).

THREADER (Jones et al., 1992) was used for threading calculations with an updated version of the fold library. The initial calculations were always done using the sequence information alone. Additional runs were done by taking into account the sequence and confidently predicted parts of the secondary structure (Jones, 1999). Interesting hits were sorted either based on their cumulative Z-scores or individual pairwise energies. In both cases, three to five Sm proteins (Kambach et al., 1999), including structurally related SMN protein (Selenko et al., 2001), were among the top 10 matches.

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